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CONTENTS

Page.

1. Foreword 1
2. Studies on Digestive Enzyme in Whale Shichiro Akiya..... 3
3. Studies on Tryptase from a Whale
   Shichiro Akiya, Yasutaro Ishikawa,
   Setsuzo Tejima, Toyohiko Tanzawa..... 8
4. Studies on the Utilization of Whale meat by the use of
   Pancreatic Tryptase of Whales
   Shichiro Akiya, Setsuzo Tejima, Yasutaro Ishikawa.....11
5. Test Culture of some Microorganisms with Whale-Meat Peptone.
   Shichiro Akiya, Fumiharu Kobo.....15
6. Chemical Studies on the Freshness of Whale Meat. I.
   Tadashi Nakai.....17
7. Chemical Studies on the Freshness of Whale Meat. II.
   Tadashi Nakai.....27
8. On the Simultaneous Extraction of Vitamin A D and Vitamin
   B_2 Complex from the Liver of a fin Whale
   Tadashi Tawara.....31
9. Studies on Whale Blood. I. Tadashi Tawara.....38
10. Sinus-hairs of the Sei Whale
    Teizo Ogawa, Junnosuke Nakai, Toshitomo Shida.....41

FOREWORD

The establlishment of Whale Reserch Institute (Juridical Foudation) was permitted by the Japanese Government in September 1946 but the research itself had been carried on for a long time prior to this. The present Institute owes its founding to the late Mr. Ikujiro Nakabe (President of the Taiyo Whaling Company) and Mr. Kenkichi Nakabe (the former Vice-President of the Taiyo Whaling Company), who found Nakabe Research Institute in 1941 in order to carry out researches concerning whales and for the future development of the whaling industry. However, the outbreak of war shortly after the establishment of the Institute made it virtually impossible to carry out researches to any extent.

The confusion of social conditions at the time of surrender made it imperative for the Institute to be disbanded, for the time being, at least. Nevertheless; individual researchs were carried on, without the permission of the Japanese Government since it would be a great loss to all concerned if all the facilities and valuable reserchers were to be dispersed.

Japan's recovery went ahead at a far greater pace than was imagined at first due to excellent administration of the Occupational authorities. With regard to whaling, not only that near the coast of Japan but whaling in the Antarctic has been granted to the Japanese so that researches in whales could once more be carried out without difficulty.

Accordingly, permission was granted by the Japanese Government to set up a juridical foundation and the Whale Research Institute was firmly established to carry on the work. Publication of results of research will be made twice a year and it is hoped that exchange of date on research in whales will be made in the near future with various colleagues all over the world.

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Studies on Digestive Enzyme in Whale

Shichiro Akiya and Setsuzo Tejima

I. Introduction

In this country where medicinal digestive enzymes are lacking due to shortages of land animals, materials for such use must be sought elsewhere. Luckily, Japanese is surrounded on all sides by the sea where resoures for marine product abounds and to which we can turn for material. It is natural, therefore, that we should turned to one of its biggest animal, the whales, not only for madicinal material but also for the solvation of our foodstuff problem.

Although biological researches on whales have been made to a large amount in the past and many bibliographical data are available, very few references can be found of chemical studies of whales, either in fundamental chemistry or in its application. The author, therefore, undertook to make some chemical experiment with whales with the object of obtaining fundamental knowledge as basis for their utilization. The first report, herewith, present some observations obtaind regarding digestive enzymes contained in whales.

II. Digestive enzymes of a whale (Balaenoptera borealis Lesson)

Researchs into pepsin like substance.

Stomachs of whales differ in structure according to species but they are generally divided into 4 sections. For the sake of convenience, the author and others named then as follows: 1st stomach the one directly connected with cesophagus. 2nd, 3rd and 4th stomachs-stomachs connected to the 1st in that order.

In order to determine how pepsin is distributed in each stomach, mucous membrane of the stomach was separated from its sinews and its dried powder was prepared.

1) No enzyme was found in the 1st stomach, very little in the 2nd, and large amount were found in the 3rd and 4th stomachs.
2) Contrary to the existing idea of protein decomposing enzyme of animals, pepsin, which acts in acid medium, the substance found in whales work best in neutral or alkaline media.

3) Certain concentrated product can be obtained from the mucuous membrane of the stomach by a simple process.
4) The substance is activated by common salt.

EXPERIMENTAL

1) Preparation of the material

Mucous membrane from each stomach of a whale (Baleanoptera borealis Lesson) dissected at Ayukawa in Miyagi prefecture, were soaked in acetone and sent to Tokyo. The concentration of acetone was about 50%. Time elapsed in transportation to Tokyo was about 10 days. The membrane was then cut into small pieces with scissors, put through a mincing machine once and soaked in two to three volumes of acetone. This was left for one hour with occasional shaking and then filtered by suction. This procedure was repeated 3 to 4 times. The membrane was finally shaken with an equal mixture of acetone and ether for dehydration and degreasing, filtered, dried in the open and finally dried in vaccuum desiccator over calcium chloride. The dried powder here obtaind was ground in mortar and put through a sieve.

2) Determination of suitable pH for activity

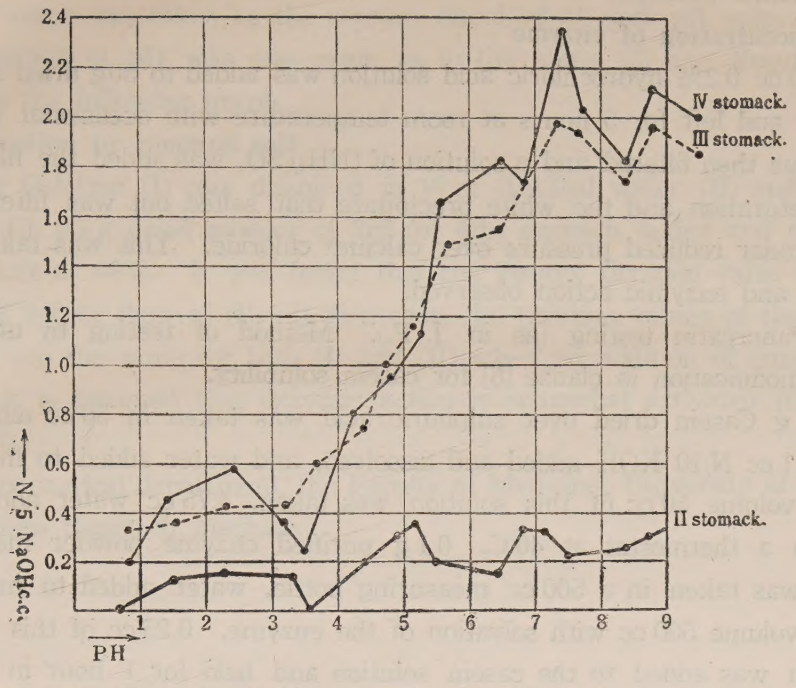
a) Determination of suitable pH by the liquefaction of gelatine.

3 g gelatine was added to 10 cc each of $^{N}/_{10}$ buffer solutions[1] of different pH values, dissolved at 50°C and placed in a thermostat of 40°C. 0.5 g of the above dried stomach powder was added to each gelatine solution and kept for 4 hours in a thermostet at 40°C. The gelatine solutions were taken out, cooled by water and their solidification observed.

The protein decomposing enzyme present in stomach is called pepsin which, as is well known, is active in acid medium of pH 1 to 2. According to bibliography, the most suitable pH values for pepsin to work on gelatine is at pH 2—3. However, according to the results obtained in the above experiment, it takes 48—72 hours for the powder of 3rd and 4th stomachs to liquefy gelatine if the pH is kept at 2 to 3. On the other hand, if the pH is maintained at 5.0 to 6.2, powder from 3rd and 4th stomachs will liquefy gelatine completely in 4 hours. Liquefaction occurs even at ph 8.5.

1) For buffer solution, Sörensen's hydrochloric acid sodium hydroxide, and Michaelis acetic acid-sodium acetate, were used. (Experimental Chemistry, II, 12, p. 127)

Table 1



b) Determination of suitable pH value by titration

0.5 g Gelatine was added to 10 cc of hydrochloric acid or sodium hydroxide of various strengths, melted at 50°C and put into thermostat at 40°C. 0.1 g Dried stomach powder was added to each of these solutions, held for 4.5 hours at 40°C in the thermostat. They were then taken out, 5 cc Formol solution[2] added and titrated with N/5 NaOH solution, the end point being coloration of the solution to red. Titration under identical conditions were made with each of the solution of various acid or alkaline gelatine without the addition of enzyme and the difference in the two values were taken as the increase in acidity at each pH value. Following graph shows the titration values at various pH values. No change in pH value was observed before and after the reaction. pH values were determined by the test paper of Toyo Filter paper Co.

On the other hand, Biuret reaction was tested with the reaction mass after reaction and obtained results coinciding well with the results of Formol titration showing deep blue coloring on acidic side and light pink in neutral

2) 1 cc 0.5% phenolphthalein (0.5 g phenolphthalein dissolved in 100 cc 50% alcohol) added to 50 cc formalin (J. P.) and made into a pink solution with N/5 NaOH solution. (Biochem. Z. 7 (1907), 45 : Z. phys. C. 64 (1910), 120.

and alkaline portions.

3) **Concentration of enzyme**

500 cc 0.2% hydrochloric acid solution was added to 50g dried stomach powder and left for 5 hours at room temperature with occasional stirring. This was then filtered and a solution of $(NH_4)_2SO_4$ was added the filtrate to 50% saturation and the white precipitate that salted out was filtered and dried under reduced pressure over calcium chloride. This was taken as a sample and enzymic action observed.

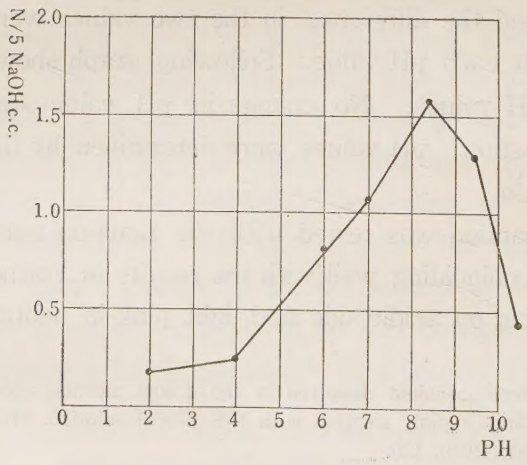
a) Pancreatin testing (as in J. P.). Method of testing by means of modification in clause [6] for casein solubility.

0.1 g Casein dried over sulphuric acid was taken in 50 cc measuring bottle, 1 cc N/10 KOH added and dissolved, and water added to make the whole volume 50 cc of this solution was taken, 4.75 cc water added and held in a thermostat at 40°C. 0.1 g purified enzyme powder mentioned above was taken in a 500 cc measuring bottle, water added to make the whole volume 500 cc with solvation of the enzyme. 0.25 cc of this enzyme solution was added to the casein solution and held for 1 hour in a thermostat at 40°C. This was then taken out and 3 drops of acetic acid alcohol solution (1 cc acetic acid, 9 cc water and 10 cc alcohol) was added but no opalescence or precipitation occured. This shows that at 40°C and at pH 7, the enzyme digests 200 times its quantity of casein in 1 hour.

b) Determination of suitable pH value

50 mg of the above mentioned salted-out enzyme was taken in a 25 cc measuring bottle and dissolved in 50% glycerine. 1 cc of this solution was taken as the enzyme solution. For a base material, 10 cc 6% gelatine solution was made into a 14 cc solutions with the addition of a suitable amount of N/5 NaOH or N/5 HCl with further addition of water. The enzyme solution was added to each of the acid or alkaline test solutions, reacted for 1 hour at 40°C and titreted with Formol solution. As a control, a solution with 1 cc of

Table 2



distilled water instead of enzyme solution was used and the difference in titration value was taken as the amount dissolved at each pH value. Determination of pH was mas made as in the above (2) b). Results are shown in the following graph.

4) **Activation by common salt**

0.5 g Gelatine (I) was dissolved in 10 cc distilled water (II) and 10 cc 0.3% NaCl, 0.1 g dried powder of 3rd (or 4th) stomach added and reacted for 4 hours at 40°C. It was found that the Formol titration value of (II) was 30% larger than of (I). In this case, the titration values of the basic material are the same for both (I) and (II) when no addition of enzyme is made. It is assumed that enzymic action is somewhat activated by commom salt.

(Pharmaceutical department, the Faculty of Medicine, University of Tokyo and Whales Research Institute.)

Studies on Tryptase from a Whale (Balaenoptera Borealis L.)

Shichiro Akiya, Yasutaro Ishikawa, Setsuzo Tejima and Toyohiko Tanzawa

In order to investigate the presence of protein decomposing enzyme in the pancreas of a whale (Balaenoptera borealis Lesson), the pancreas was dehydrated and degreased with acetone and other, dried and prepared into a powder. Following results were obtaind.

1) The powder obtained by grinding the dried powder in the mortar and putting it through No. 5 sieve can pass the test for pancreatin described in Par. 6 of Japanese Pharmacopoeia.

2) The most suitable pH value for gelatine is in the range of pH 8—9 (Fig. 1) and the most suitable temperature (for 1 hour duration of activity is 50°C.)

3) When the amount of base material is constant and the amount of enzymes varied, the activity curve (Fig. 2) shows that the amount of enzyme and actiyity are proportionate.

4) The activity curve (Fig. 3) obtained by holding the amount of enzyme, material, pH and the temperature of activity at a constant and varying the time of activity, shows it increasing up to 4—hour period.

5) Distribution of tryptase in various parts of pancreas did not vary greatly between those in tail, body and head parts, but the amount in the tail part was 110% of the total of other two parts.

The purification of pancreatic tryptase is to follow the preparative methods for chymotrypsin and trypsin crystals from bovine pancreas as reported by Northrop. Prepartions are now being made for carrying out the experiments. Although no definite report can yet be made, the authors are convinced that if bovine pancreas is used, crystals of chymotrypsinogen and trypsinogen can be prepared comparatively easily by the Northrop method.

EXPERIMENTAL

1) Preparation of enzyme powder

Pancreas obtained from a whale (Balaenoptera borealis Lesson) dissected at Ayukawa in Miyagi Prefecture was immersed in twice its volume of

acetone for 10 days, taken out, finely cut with acissors and put through a mincing machine. To this was added 2 to 3 volumes of acetone, drained, and the process repeated 3 to 4 times to dehydrate and degrease. Finally, the residue was shaken with an equal mixture of acetone and ether, filterd, in the open and then dried in a vacuum desiccator over calcium chloride. From 2500 g of pancreas, 300 g of dried substance were obtained. This substance was ground in a mortar and put through a No. 5 sieve (J. P.). Yield of the powder to the dried substance, ca 40%.

2) **Pancreatin Test, Par. 6, Japan Pharmacopoeia**

0.1 g Enzyme powder prepared as in the above (1) was taken in a 500 cc measuring bottle distilled water added to make the total volume 500 cc and filtered. 2 cc of the filitrate was dissolved in 1 cc N/10 KOH and distilled water added to make the total volume 50 cc, 3 cc of this solution was added to 2 cc distilled water, reacted for 1 hour at 40°C, after which 3 drops of acetic acid alcohol solution (1 parts glacial acetic acid, 9 parts water, 10 parts alcohol were added. Only slight opalescence could be seen.

3) **Determinnation of suitable pH of activity**

3 g Enzyme powder as described in (1) was added to 30 cc 80% glycerine and left standing over-night at room temperature. A clear upper solution was separated centrifugaly, 9 times its volumeo of distilled water was added and 1 cc of this solution was used as enzyme sample. For the base material, 0.5 g of gelatine was added to 10 cc each of glycocoll-NaOH buffer solution (Sörensen's) of varying pH values, and melted at 50°C. To this was added 1 cc enzyme sample at 40°C, held there for 2 hours and the amino acids thus formed titrated, in Willstätter's alcohol solution, with N/5 alcoholic KOH solution with thymol-phthalein as an indicator. On the other hand, the base with 1 cc of distilled water instead of the enzyme powder was titrated and the difference between the two values were taken as the degree of digestion at that pH value. Results are shown in Fig. 1.

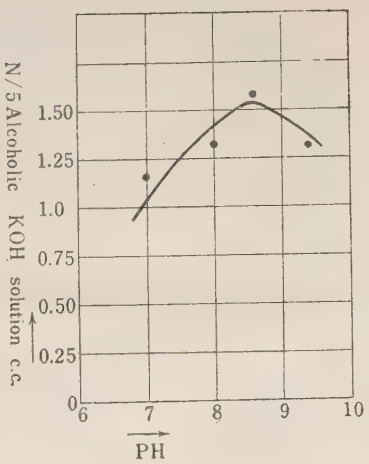


Fig. 1

4) **Activity curve by the change in the amount of enzyme**

0.5 cc, 1 cc, 2 cc, 3 cc, 4 cc and 5 cc of the glycerineextract of pancreas, as described in (3), were added to solutions in which 0.5 g gelatine was dissolved in 10 cc NaOH-glycocoll buffer solution of pH 8.1 and water added to make the total volume 15 cc. This was reacted for 2 hours at 40°C and finally titrated as in (3). Results are shown in Fig. 2.

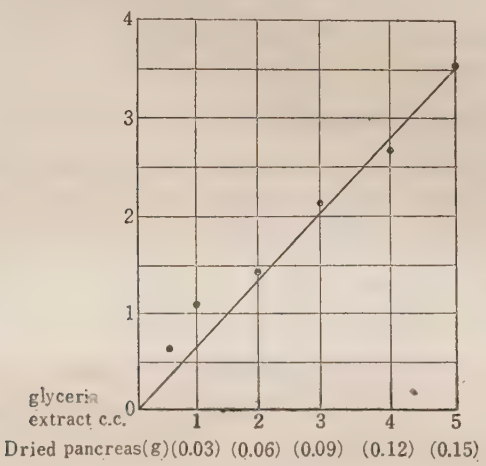


Fig. 2

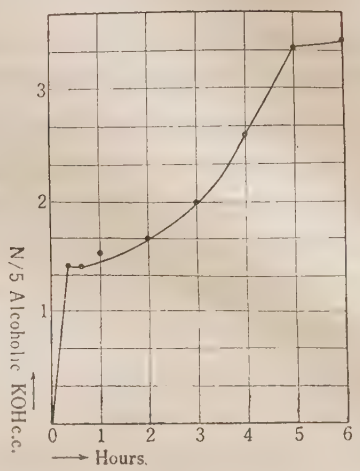


Fig. 3

5) **Activity curve for varied action time**

2 cc glycerine extract of pancreas as described in (3) was dissolved in 10 cc solution of NaOH-glycocoll buffer solution of pH 8.1 in which a base of 0.5 g gelatine was dissolved. The reaction temperature was kept constant at 40°C and time varied at 15 minutes, 30 minutes, 1, 2, 3, 4, 5 and 6 hours. Titration was performed as in (3). Results are shown in Fig. 3.

(Pharmaceutical department, the Facluty of Medicine, University of Tokyo and Whales Research Institute.)

Studies on the Utilization of Whale Meat by the use of Pancreatic Tryptase of Whales

Shichiro Akiya, Setsuzo Tejima and Yasutaro Ishikawa

The present experiments were carried out in order to obtain enzymes suitable for bacterial culture and production of diphtheria antitoxin from whale meat digested by whale pancreas. The authors prepared 25 kinds of peptone from a definite quantity of meat at a definite pH value with varying amounts of enzyme and at different lengths of time. With each of the peptone obtained, the ration of total nitrigen to free amino radical-N and the yield of peptone were measured. With each peptone obtained under various conditions, test culture of diphtheria bacillus and production of its antitoxin were tested. As a result of these experiments, following observations were made:

1) It was found that better yields of peptone were obtained when reaction time was prolonged. For example, when 3 g of pancreactic powder was used, yield increased proportionately with increase in reaction time.

Table 1

No.	Reaction time (Hours)	Nitrogen of liquefied portion (g)	Percent of digestive (%) (Comment. 1)	Amino nitrogen : Total nitrogen
12	12	4.42	36.0	1 : 3.0
2	24	6.63	51.5	1 : 2.3
6	48	6.93	56.5	1 : 2.7
8	72	9.51	77.3	1 : 2.7
23	120	12.06	98.0	1 : 2.2

2) When the reaction time was made constant, the amount of amino nitrogen increased with the increase in the amount of enzyme but no effect

Table 2

No.	Amount of used pancreas (g)	Amino nitrogen : Total nitrogen	Nitrogen of liquefied portion (g)	Percent of digestive (%)
1	2	1 : 3.4	6.88	56.0
2	3	1 : 2.9	6.33	51.5
3	5	1 : 1.9	7.46	60.7
4	7.5	1 : 1.3	8.00	65.0

1) As is described later, the amount of nitrogen in Frozen whale-meat is 16.4%. This value was obtained by dividing the amount (in gram) of nitrogen found in liquefied portion by $0.164 \times 300 \times 0.25$ and then multiplied by 100.

was observed in the yield of peptone, as can be seen from the following graph where the reaction was limited to 24 hours.

3) Addition of duodenum powder amounting to about 1/3 the amount of panccreas powder results in the increase of 20% in the yield even when the length of reaction time is the same.

Table 3

No.	Amount of used pancreas (g)	Amount of used duodenum (g)	Reaction time (hours)	Yield (g)
13	1	0.3	24	35.5
24	1	0.3	24	43.5
2	2	1.7	24	44.0
25	2	0.7	24	50.5
2	3	1.0	24	44.1
26	3	1.0	24	55.0

4) As a result of experiments in diphtheria culture and production of antitoxin, under various, it was found that some of the peptone obtained by the authors were as good as the market products. It was also found that those suited to the production of antitoxin were those in which the ratio of total nitrogen to amino nitrogen was in the order of 3.9.1.

EXPERIMENTAL

1) **Preparation of enzyme powder**

Enzyme powder as described in the paragraph on ' Tryptase in a whale (baleanoptera borealis Lesson)' was used. This was prepared by drying whale pancreas with acetone and ether, grounding in mortar and putting through a sieve No. 5. Duodenum was also prepared in the same manner.

2) **Perparation of whalemeat peptone**

300 g of frozen whale-meat was chopped finely with a knife and put through a mincing machine. 530 cc water was added to this[2] and boiled[3] for 10 minutes. This was then cooled and placed in a thermotat at 40°C. pH value at this juncture is 5.6 which was corrected to pH 8.6 with suitable addition of caustic alkali. To this solution are added enzyme powder and a small amount of toluene as a preservative. This is then kept in a thermostat but for the first 6 hours, pH value must be corrected every 1 to 2

2) This amount of water was determined from the amount of water (75%) found in frozen whale-meat as described later, Calculation: $300\times0.25\times10-300\times0.75=530$.

3) pH values were determined with the test papers T. B., B. T. B., and C. R. of Tokyo Roshi Co.

hours because pH values tend to shift to acidic range at first. For instance, if enzyme powder is added to a solution of pH 8.6, it will shift to pH 7 after 90 minutes. After 6 hours from the time of the addition of enzyme powder, pH values do not vary greatly, so that pH values are determined only in 4 to 6 hours. By this correction, pH value is value is mainted at 8.6.[4] After a lapse of definite time, the solution is taken out of the thermostat, boiled for 10 minutes to break the enzyme, pH corrected to 4.7 with hydrochloric acid and filtered while hot through folded filter paper.[5] The residue is washed once with 300 to 400 cc of hot water and and the washing is added to the filtrate. The amount of filter and the residue are weighed, 1 cc of which is taken to determine total nitrogen by Kjeldahl method and amino nitrogen by Van Slyke method. The filtrate is neutralized with sodium hydroxide and concentrated on a steam bath as much as possible to a paste. This paste is tentatively called whale-meat peptone. The time of reaction was varied at 12, 24, 48, 72 and 120 hours, and the amount of enzyme to 1, 2, 3, 4, 5 and 7.5 g. Tests were also made in which duodenum powder to the amount of 1/3 of pancreatic powder was added at the same time and another in which 5 g dried powder of 4th stomach was used in place of pancreatic powder.

3) **Determination of the amount of water and total nitrogn in frozen whale-meat.**

5 g Frozen whale-meat forced through the mincing machine was mixed with 5 g sand and held in a thermostat at 110°C until there was no change in weight. The loss in weight was taken as the amount of water contained in frozen meat.

Weight at the end of 22 hours 1.2508 g

$$\text{Water } \% = (5 - 1.2508/5 \times 100 = 75.0\% \ (75.0\%)$$

The frozen whale-meat was dehydrated and degrease with acetone and ether, dried and the amount of toatl nitrogen determined by Kjeldahl method.

Sample 50 mg. N/10 H_2SO_4 20 cc (F=0.902), N/10 NaOH 13.87 cc (F=1.000)

$$N\% = 1.4(20 \times 0.982 - 13.78) \times 100/50 = 1.64\%$$

4) pH values were determined by the test paper T. B., B. T. B., and C. R., of the Tokyo Roshi Co.

5) If filtration is performed while hot, filtration is rapid enough even at this pH value. Filtration cannot be done at alkaline state and even at pH 4.7 it becomes diffecult if allowed to cool.

4) **Culture of diphtheria bacilli and production of antitoxin**

Whale-meat peptone No. 11 and 25 were used for the culture media for the production of diphtheria toxin. Composition of culture midium was as follows:

Horse meat bouillon[6] ..... 250 cc
Pepton ..... 2.5 g (1%)
CH_3COONa ..... 1.25 g (0.5%)
$MgSO_4$ ..... 0.05 g (0.02%)
$NaHPO_4$ ..... 0.25 g (0.1%)
$CaCl_2$ ..... 0.25 g (0.1%)

The pH value of the medium was corrected to 7.6 with NaOH, boiled for 10 minutes, filtered and the pH value of the filtrated again corrected. This solution was then poured into D-type flask of 250 cc capacity, sterilized at 110°C for minutes and 2.5 cc 20% rice syrup[7] (amount of syrup to medium: 0.2%) added aseptically. To this media was planted diphtheria bacilli, strain No. 8 of park-William's, and held for 7 hours at 37°C.

Table 4

Pepton	pH at inoculation	Growth after 7 days	pH at end time	Lf value/c.c.	Kf value
11	7.6	++	8.8	8	6
15	7.6	++	9.0	7	6
Carno	7.6	++	9.0	7	6

Finally, authers deeply thank Prof. Shogo Hosoya, Prof. Michizo Asano and Mr. Goro Urakubo of Infectioins Disease Institute of Tokyo University.

(Pharmaceutical department, the Faculty of medicine, University of Tokyo and Whales Reserch Institute.)

6) Preparation of horse-meat bouillon:
Finely chopped horse-meat was added to 5 times its amount in weight of 5% acetic acid, kept for 30 minutes and the pH corrected to 5.8 with 20% NaOH. This was boiled for 10 minutes and filtered.

7) Preparation of rice syrup:
Market product of rice syrup was dissolved in water to obtain 20% solution, poured into test tubes and left for 1 day. They were then disinfected at 100°C for 15 minutes, intermittently.

8) Signs used to designate the amount of growth:
- Bacterial film not covering more than half of the surface after 7 days.
+ Bacterial film covering more than half but not all of the surface after 7 days.
++ Bacterial film covering the whole surface after 7 days.
+++ Bacterial film covering the whole surface and constituting a layer.

The Test Culture of some Microorganisms with Whale Meat Peptone

Shichiro Akiya and Fumiharu Kobo

This experiment was carried out to determine the difference of value of utilization between the samples and market-peptones. Market peptones were used as follow;

Teruuchi's, Witte's, Carneau's and Chapteaut's Peptone.

Experimental

Incubation-condition;

Culture media; Peptone-water prepared with the some kinds of peptones as above descrived.

Test-strains; B. coli communis, B. typhi, Pneumo coccus, B. dysenterie Minoda, V. cholerae, B. subtilie M. B. 25, Staph. coccus aureus Terajima, B. pyocyaneus.

Incubation; 37°C., 24 hrs.

Result;

The amount of increased bacillus, the formation of dyestuff and H.S are shown as follow:

B. Coli communis

Peptone \ Hours	6	12	18	24	Order	Indol-formation			
						Ehrlich's Method		Kitazato's Method	
Sample	+	+	++	+++	1	++	2	+	5
Teruuchi	++	++	++	+++	2	+++	1	++	3
Carneau	+	++	++	+++	3	+	5	++	2
Witte	±	+	+	++	5	++	4	++	4
Chaptoteaut	+	++	++	++	4	++	3	+++	1

B. typhi

Peptone \ Hours	6	12	18	24	Order
Sample	+	++	+++	+++	2
Teruuchi	+	++	+++	+++	1
Carneau	+	++	++	++	4
Witte	+	+	+	++	5
Chaptoteaut	+	++	++	+++	3

Pneumococcus 1

Peptone \ Hours	6	12	18	24	Order
Sample	++	+++	+++	+++	4
Teruuchi	+++	+++	+++	+++	1
Carneau	++	+++	+++	+++	5
Witte	++	+++	+++	+++	3
Chaptoteaut	+	+++	+++	+++	2

B. dysentheriae Minoda

Peptone \ Hours	6	12	18	24	Order
Sample	+	+	+	++	4
Teruuchi	++	++	++	+++	1
Carneau	+	++	++	+++	2
Witte	±	+	+	++	5
Chaptoteaut	+	++	++	++	3

V. cholerae O.

Peptone \ Hours	6	12	18	24	Order	Indol-formation	Order
Sample	++	++	++	+++	1	+	5
Teruuchi	+	+	++	+++	4	+	4
Carnean	++	++	++	+++	3	+	5
Witte	+	+	++	++	5	+	2
Chaptoteaut	±	+	+	+++	2	++	1

The experiments above described led summarily to the results as follow :

Peptone	Order of Bac.-growth					Order of Indol-formation		
Sample	1	1	2	4	4	2	3	5
Teruuchi	1	1	1	2	4	1	3	4
Carneau	2	3	3	3	3	2	5	5
Witte	2	5	5	5	5	2	4	4
Chaptoteaut	2	2	3	3	4	1	1	3

(i) The amount of increased Bac. Incubation ; 24 hrs, Peptone-water, pH 7.0

Peptone \ Strain	B. subtilis M. B. 15	Order	B. pyocyaneus	Order	Staph. aureus Terajima	Order
Teruuchi	+++	1	+++	1	+++	1
Witte	++	4	++	2	+++	1
Sample	+++	2	+++	1	+++	1
Carneau	+++	3	+++	1	+++	1
Chaptoteat	+++	3	+++	1	+++	1

(ii) B. typhi ; H_2S-Formation tested by Lead-acetate-paper. pH 6.8

Peptone \ Hours	24	36
Teruuchi	+++	+++
Witte	+	++
Sample	−	−
Carneau	++	+++
Chaptoteaut	−	−

(iii) Formation of dyestuff and Indol. B. Pyocyaneus. Incubation ; 36hrs, pH 7.0

Peptone \ Reaction	Dyestuff	Indol-formation: Ehrlich's Method	Indol-formation: Kitazato's Method	Growth
Teruuchi	−	+++	+++	+++
Witte	+++	+	+	++
Sample	−	++	+	+++
Carneau	++	++	+	++
Chaptoteaut	+	+	+	+++

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(Pharmaceutical department, the Faculty of Medicine, University of Tokyo and Whales Research Institute.)

Chemical Studies on the Freshness of Whale Meat. I.

Evaluation of freshness and changes in quantity of several kinds of nitrogen in whale meat following deterioration of freshness.

Tadashi Nakai

There is generally a lapse of time on the sea between the time when a whale is harpooned and until it is dissected. There is still a longer lapse of time before this prepared whale meat gets into the hand of consumers. It follows, therefore, that there should be more chance of deterioration of freshness in whale meat than in the meat of domestic animals and fowls. Preservation method and use of whale meat varies according to the degree of freshness. It can be said, therefore, that the question of the freshness of whale meat has the most important significance in the handling of whale meat.

The author has undertaken the present studies with the object of making chemical investigations on the deterioration of freshness of whale meat and of making a contribution to the problem of maintaining freshness.

First, it is necessary to decide on the evaluation method of the freshness of whale meat in the author's future experiments.

An example of the evaluation method used by operators in the field in Japan is shown in Table I.[1], but it is too subjective to be adopted as the method in the author's experiments, and even in the field it could only be used at the time of dissection.

Table I. Standard of Freshness of Whale Carcass.

Grade	Colour and lustre of flesh	Elasticity of muscle.	Offensive smell	Exudation of blood	Lapse of time between killing and dissection
Very fresh (90%)	Very dark red, semitransparent, lustrous	Conspicuous	none	none	Less than 6 hrs.
Fresh (80%)	More reddish, lustrous	Recognizable	〃	Recognizable by pressing	〃 12 〃
Satisfactory (60%)	Reddish grey, non-lustrous	none	〃	Recognizable	〃 18 〃
Unsatisfactory (40%)	〃	〃	Recognizable	Conspicuous	〃 24 〃
Poor (Less than 40%)	〃	〃	Extreme	Flowing	More than 25 hrs.

Chemical methods for determination of the quality of flesh have been worked out by many researchers already and among them, the method based

on the determination of various kinds of nitrogen has been studied most and it seems to be more reliable than the others, as for instance, oxygen consumption test, methylene blue and nitrate redution test and hydrogen sulfide test.

According to Malin[2], high-quality meat, fish and fowl shows 6—16 mg, satisfactory fish and fowl, 18—26 mg and poor quality meat, fish and fowl, 27 or more mg total NH_3 per 100 g.

Tillmans and Otto[3] reported that at the beginning of the decomposition of fish, NH_3 reached 30 mg and amino acid N 100 mg per 100 g, and Shimizu[4] found ammonia N 30—40 mg and amino acid N 80 mg per 100 g at the time.

Glassmann and Rochwarger[5] reported that 0.020% and 0.025% are the respective critical quantity of NH_3 indicating the beginning of putrefaction in the flesh of warm-blooded animals and of fish.

Herzner and Mann[6] observed that the amount of pure albumin in meat, which decreases with the progress of putrefaction, gave an indication of incipient putrefaction and that a pH of over 6.2 indicated putrefaction in meat.

Ipatov[7] recommended the determination of hydrolized protein for evaluation of the freshness of meat.

For measurement of spoilage in fish, Beatty and Gibbons[8] recommended the determination of trimethyl amine fraction in the filtered muscle juice. They found that fresh muscle showed a mean value of 0.17 mg of trimethyl amine N in each 100 cc of muscle juice; that odors appeared at about 4.0—6.0 mg and that the trimethyl amine fraction did not increase appreciably by autolysis.

For detecting incipient decay in meat, Brotzu[9] recommended the determinations of amino acid N and pH of meat juice which transuded from minced meat by heating, and of the bacterial count of juice obtained by pressing the minced meat. He found that 300—350 mg of amino acid N (Sörensen) per 100 cc of meat juice and pH 6.3 and above indicated incipient decomposition.

According to Yamamura[10] there is a straight linear relation between ammonia content and pH of fish. He observed that ammonia content at the beginning of putrefaction was 30 mg per 100 g, as was found by Tillmans and Otto, and the colorimetric measurement of pH at this period

was 6.5.

The present paper embodies the author's observations on the changes in the amount of some nitrogen in whale meat during the course of deterioration of freshness, which have been carried out with the object of determining of nitrogen which would indicate the degree of freshness in whale meat.

I. Changes in the amount of volatile basic N, non-protein N, formol titrating N, trimethyl amine N and trimethyl amine-oxide N were determined in minced whale meat standing at room temperature.

The material used in the experiment was the 'oniku', a portion of flesh between the dorsal fin and tail, of blue whale (shironagasu-kujira; *Balaenoptera musclus*). Its fat content was very high, namely 29.2%. Samples, taken from the minced material, showed a distinct acid reaction and emitted strong sour odor all through the course of experiment. The author believes that this was due to the formation of a remarkable amount of free low fatty acids resulting from the decomposition of fat by enzyme in muscles and that, consequently, the propagation and the kind of bacteria was considerably restricted.

The amounts of volatile basic N and formol titrating N increased regularly with lapse of time, and the rate of increase was great (Table III and V). The increare of non-protein N (Table IV) and trimethyl amine N (Table V) were comparatively small. Change in the amount of trimethyl amine-oxide was practically unnoticed (Table V).

On account of the strong sour odor, it was difficult to detect the putrid odor at the incipient stage of putrefaction of protein in the sample. However, assuming that the onset of putrefaction took place when gas generation in the interior of a sample (formation of bubbles) was detected, it was found that the amount at this period were: volatile basic N, 30.2 and 29.7 mg per 100 g, non-protein N, 500 mg, formol titrating N, 136 mg and trimethyl amine N, 0.45 mg.

II. Comparison was then made of the percentage value of freshness which, as shown in Table I, is determined rather subjectively by the whale-fishing men in Japan for evaluating the degree of freshness at the time of dissection, and the quantities of volatile basic nitrogen and of formol titrating nitrogen.

The material used in this experiment was flesh of sperm whale (mak-

kō-kujira ; *Physeter macrocephalus*).

The results obtained are listed in Table VI and also summarized in Table II.

Table II

Freshness % (cf. Table I)	Volatile basic N (mg/100 g)	Formol titrating N (mg/100 g)	Number of samples used
90	3.5	61.5	1
85	5.5	70.7	4
80	8.2	81.2	5
75	11.5	90.2	4
70	16.0	95.8	1
60	38.6		1
50	246		1
40	453		1

With the progress of deterioration from 90% to 70%, the mean value of nitrogen for each percentage value shows gradual and yet a definite increase, as shown in Table II. However, there are some inconsistencies among the individual measurement values for different percentage values, as shown in Table VI.

During the course of deterioration under 70%, quuntitiy of volatile basic N shows a sudden increase. Formol titrating N during this period was not determined owing to the matter of sampling.

These results indicate that the change in the percentage values which are determined by experts, corresponds to that in the amount of volatile basic and formol titrating nitrogen.

From the results of these experiments, it has become clear that the freshness of whale meat can be evaluated by determining the volatile basic nitrogen or formol titrating nitrogen.

In author's future experiments, therefore, comparison on the degree of freshness of whale meat will be done by the comparison on the amount of volatile basic nitrogen or of formol titrating nitrogen.

Experimental

I. Deterioration of freshness of minced whale meat at room temperature.

a) Change in the quantity of volatile basic nitrogen.

Material and treatment :— The material used was the refrigerated 'oniku,' the portion of flesh between the dorsal fin and tail, of blue whale (shironagasu-kujira , *Balaenoptera musclus*) caught in the Antarctic Occan.

Analytical data of the material are shown in the following table.

Moisture	Crude protein	Crude fat	Ash
54.70%	15.58%	29.20%	0.64%

The minced, pounded and thoroughly mixed material was devided into many Petri' dishes. All the dishes were put in an air-tight glass vessel containing a small amount of water in the bottom and kept at room temprature. After standing for a definite time, a dish was taken out from the vessel for a determination, one dish for each value. The amount of a in sample in one dish was about 50 g. Temperature at noon during the course of experiments, 20°--22°C.

Method of determination:— The determination was made by the aeration method.[11),12)] 4 g of thoroughly mixed sample was introduced into a test-tube, and 20 cc of distilled water, 0.5 g MgO, 0.1 g NaF, and a few drops of octyl alcohol to prevent foaming, were added. By means of a fairly rapid flow of air current throtgh the mixture at 40°C by suction, volatile basic substance was driven out into 10—15 cc of 0.02 *N* H_2SO_4. After 3 hrs' continued aeration, excess acid was titrated with 0.02 *N* NaOH with methyl red-methylene blue indicator.

Results obtained are shown in Table III.

Table III.

Time (hrs)	Volatile basic N (mg/100 g)	Indications	Time (hrs)	Volatile basic N (mg/100 g)	Indications
0	13.2		165	35.7	
21	17.3	Faint sour odor.	189	38.4	Distinct gas evolution and putrid smell.
45	20.3	Strong sour odor.	213	40.5	
69	23.6		262	47.1	
93	27.2		310	50.3	
117	30.2	Sour odur, slightly diminished Gas generation in the interior.	381	53.7	
141	33.0				

b) Change in the amount of non-protein nitrogen.

Material and treatment:— Same as in a). Temperature at noon during the experiment, 20°—22°C.

Method of determination:— 5 g of thoroughly mixed sample was weighed into a mortar, thoroughly pounded with 0.5 g of emery powder, transferred to a beaker, washed out with 50 cc of distilled water and 5 cc of ether and heated on a steam bath for 10 min. under occasional stirring.

25 cc each of Barnstain's $CuSO_4$ solution and NaOH solution were added and after standing for 30 min., supernatant liquid was decanted on filter-paper, collecting the filtrate in 500 cc volumetric flask. The precipitate was washed with 50 cc distilled water, and, after standing for 15 min., the liquid was decated as before. After the washings were repeated using six 50 cc portions of water, the combined filtrate and washings were diluted with distilled water to the volume indicated and mixed thoroughly. 10 cc of this solution was used for the determination of nitrogen by the Kjeldahl method.

Results obtained are listed in Table IV.

Table IV.

Time (hrs)	Non-protein N (mg/100 g)	Indications	Time (her)	Non-protein N (mg/100 g)
0	460		116	539
20	469	Faint sour odor.	140	562
44	478	Distinct sour odor.	190	611
68	497		237	642
92	500	Sour odor and faint putrid smell.		

c) Changes in the amount of formol titrating N, volatile basic N, trimethyl amine N, and trimethyl amine-oxide N. Material and treatment:— Material used was the same as in a) and b), but somewhat inferior in freshness. Treated as above. Temperature at noon during the experiment, 29°—30°C.

Method of determinaton:—

Formol titrating N— 100 g of throughly mixed sample was weighed into a beaker, 300 cc of distilled water added, and heated in a water bath for 20 min., under stirring. After standing for a short time, the liquid was decanted into an Erlemeyer's flask, and liquid was squeezed out of the meat residue in the beaker with a piece of gauze. The residue was extracted three times with successive 200 cc portions of distilled water as in the foregoing. To the combined turbic liquid in the flask was added 10 cc of 20% sulfosalicylic acid, and after a few hours, the mixture was filtered through a moistened filter paper, collecting the filtrate in 1 *l* volumetric flask. The residue on the filter was washed with 0.2% sulfosalicylic acid solution until the combined filtrate and washings reach the marking on the flask. The solution thus obtained is hereafter called solution [A]. 20 cc of the solution [A] was submitted to formol titration (A. O. A. C.—method[13]) with 0.2 *N* $Ba(OH)_2$.

Volatile basic N— 20 cc of the solution [A] was neutralized with 0.2 *N* $Ba(OH)_2$, 0.5 g of MgO and a few drops of octyl alcohol added. and the mixture was put through the aeration method as in a).

Trimethyl amine N— There are many worker's reports on the determination of trmethyl amine, but complicated and slow method, even if it is accurate, cannot be used in follwing the deterioration of freshness. Determination in the present experiment was carried out according to the author's modification of Lintzel's method[14] used for humann urine. This method is based on the fact that ammonia combines with formaldehyd but trimethyl amine does not and is driven out by aeration. Beatty and Gibbons[8] reported a determination method based on this principle for the spoilage in fish.

To 500 cc of the solution [A] was added 15% NaOH solution to render it weakly acid to litmus paper. The solution obtained was evaporated to 40—50 cc volume on a steam bath. To this were added 15 cc of 35% formalin, 15% NaOH until slightly acid to litmus paper, and then, 2 g of MgO and a few drops of octyl alcohol. The mixture was then immediately submitted to aeration, continuing for 4 hrs. at 40°C, into 5 cc of 0.02 *N* H_2SO_4. Excess acid was titrated with 0.02 *N* NaOH with methyl red-methylene blue indicator.

Trimethyl amine-oxide N— The determination was based on the Hoppe-Seyler's method, but aeration was substituted for distillation in the said method.

200 cc of the solution [A] was heated for 1 hr. on an asbestos-wire-netting with 10 cc of hydrochloric acid, and was rendered weakly acidic with NaOH solution. The solution obtained was evaporated on a steam bath to 20—25 cc. 0.5 g of MgO was then added, and by means of a rapid current of air through the mixture at 40°C for 4 hrs., volatile basic substance was driven out. The remainder was centrifuged, and the supernatant clear solution was seperated into a beaker. The precipitate was washed 3 times by centrifuge with successive 5 cc of distillred water. To the all combined supernatant clear solution in the beaker were added hydrochloric acid until slightly acid to congo red paper and then 10 cc of 40% $SnCl_2$. The mixture was heated on an asbestos-wire-netting for 20 min., and then neutralized with NaOH solution. After the addition of 0.5 g MgO to the mixture, trimthyl amine derived from the oxide by reduction was driven out into 0.02 *N* H_2SO_4 by aeration at 40°C for 4 hrs. Excess

acid was titrated with 0.02 *N* NaOH.

From the above experiments, the following results were obtained.

Table V.

Time (hrs)	Volatile basic N*	Formol titrating N*	Trimethyl amine N*	Trimethyl amineoxide N*	Indications
0	20.3	90.6	0.34	1.33	Dark tinge. Faint sour odor.
20	22.8	111	0.35		Distinct sour odor.
44	29.7	136	0.46	1.16	Strong sour odor. Gas generation in the interior.
68	34.9	163			
92	41.8	183	0.57		
140	50.2	216	0.70	1.34	
188	52.9	238			Putrid smell.
236	58.9	260	0.84	1.30	

(* in mg/100 g)

II. Comparison between percentage value of freshness of whale meat at the time of dissection and the amount of volatile basic and formol titrating nitrogen.

Material and tratment :— 18 pieces of meat taken from 9 sperm whales which were caught off the coast of Kinka-zan in August and Ocotver, 1947 were used.

The material was taken from the animal being dissected, and the percentage value of freshness of the material was determined by a practised operator immediately. Without delay, the material was minced and thoroughly mixed. As soon as possible, 4 g of the minced material was weighed into a test-tubs, and 8 cc of absolute alcoholic 0.01 *N* H_2SO_4 was added as a preventive for subsequent decay. Samples thus obtained were taken to laboratory, and the amount of nitrogen determined.

Method of determination :—

Volatile basic N— The sample was thoroughly pounded in a mortar, transferred to the aeration apparatus, using 15 cc of distilled water, and the aeration performed as in a).

Formol titrating N— 40 cc of distilled water was added to the sample in a beaker, and the mixture was heated in a gently boilling water bath for 15 min. under occasional stirring. The liquid was decanted throgh a filter into an Erlenmeyer's flask, and the residue in the beaker washed 3 times with succesive 20 cc portions of distilled water with heating in a water bath for 5 min, each. 2 cc of 20% sulfosalicylic acid was added to

the combined filtrate and washings in the flask. The mixture was allowed to stand overnight, and filtered into 100 cc volumetric flask. The residue on the filter was washed until the volume of combined filtrate and washings reached the marking. Formol titration was performed using 40 cc of the solution obtained.

Results obtained ware as follows:—

Table VI.

Freshness %	Volatile basic N*	Formol titrating N*
90	3.5	61.5
	4.1	69.5
	2.2	62.8
	8.1	71.1
	7.7	79.5
	average 5.5	average 70.7
80	10.6	87.4
	6.2	79.1
	7.6	79.1
	8.6	72.8
	7.9	87.4
	average 8.2	average 81.2
75	10.2	98.8
	9.9	82.2
	8.5	77.8
	17.2	102.1
	average 11.5	average 90.2
70	16.0	—
60	38.6	—
50	246	—
40	453	—

(* in mg/100 g)

In conclusion the author wishes to express his sincere gratitude to Dr. Tsutomu Maruyama for his kind guidance given during the course of the work and his inspection of the manuscript. The author also wishes to express his gratitude to Prof. Dr. Shichiro Akiya and Mr. Hisashi Okuda for their many kind advices during this experiment. Further, the author wishes to thank Messrs. R. Nagaura, T. Ishimori, T. Nishihara and S. Itani who afforded him facilities for obtaining material for the experiment. To Mr. T. Tawara and Mr. R. Fukazawa the author is indebted for their kind assistances during the work.

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(Feb. 4, 1948)

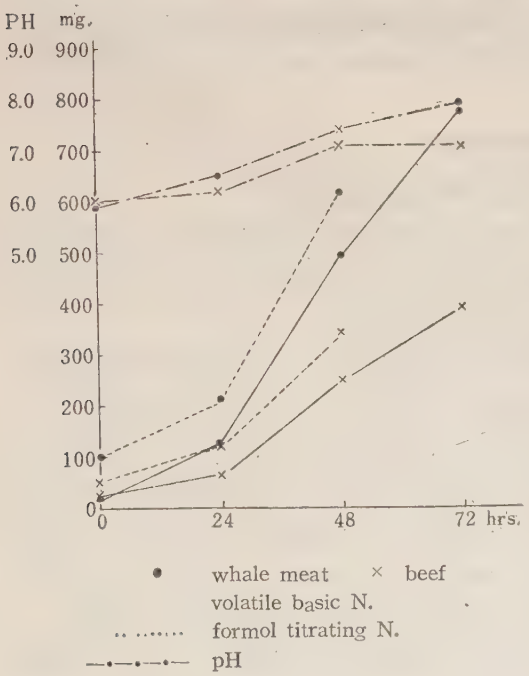
Chemical studies on the Freshness of Whale Meat. II.

On comparison between whale meat and beef
on deteioration of freshness and autolysis.

Tadashi Nakai

In the previous paper (this bulletin, p. 17) the author reported that the freshness of whale meat can be evaluted by determining the volatile basic nitrogen, i. e. ammonia nitrogen, or formol titrating nitrogen, as various workers have done on other kinds of meat. Although it cannot be said that the freshness of whale meat and that of other kinds of meat can be compared by the absolute quantity of these nitrogen in a definite quantity of meat, yet taking into consideration the fact that the volatile basic substances and the formol titrating sudstances are products in every stage of deterioration of freshness or putrefaction, the author believes that a comparison of the readiness of deterioration of freshness or putrefaction in whale and other meat can be made by comparing the condition of increase in the amounts of the two kinds of nitrogen with lapse of time. For this purpose, a piece of flesh of sei-whale (Iwashi-kujira ; *Balaenoptera borealis*) and a piece of beef were stood at room temperature in summer and changes in the amount of volatile basic nitrogen, formol titrating nitrogen and a change in pH value with lapse of time were determined. Results obtained are listed in Table I and summarized in Fig. 1. As shown in Fig. 1, the amounts of these nitrogen, especially that of volatile basic nitrogen, makes a greater increase during the course of the deterioration of freshness in sei-whale than in beef. Formol titra-

Fig. 1.

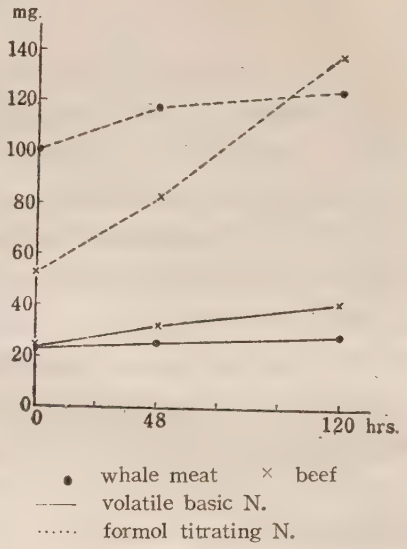


ting nitrogen in sei-whale meat was far greater than beef in absolute quantity but the rate of increase in beef was slightly above that of sei-whale meat. Increase of pH value was more rapid in sei-whale meat than in beef. This may be due to a large production of volatile basic substances in sei-whale meat.

From these results, it can be said that the freshness of sei-whale meat is more readily deteriorated than that of beef, provided that the kind of bacteria attacking meat is not brought into question.

At the same time, autolysis which plays a part in the deterioration of freshness in company with bacterial action was compared in both meat. The results obtained are shown in Table II and summarized in Fig. 2.

Fig. 2



Increase in the amount of nitrogen resulting from autolysis was found to be very small compared to that found when in united action with bacteria which mentioned above. Increase of both kinds of nitrogen was found to be greater in beef than in sei-whale meat during the process of autolysis.

Experimental

a) Deterioration of freshness at room temperature.

A piece of sei-whale meat preserved in an ice chamber for three days after dissection and a piece of beef stored in a refrigerator by a butcher were used as materials in the present experiments.

Each material was divided about equally into several Petri' dishes and let stood at room temperature in August. Every 24 hrs. the content of a dish for each material was minced and thoroughly pounded in a mortar. 4 g of each meat thus treated was weighed into a test-tube and 6 cc of absolute alcoholic 0.01 N H_2SO_4 was added to prevent subsequent decay. Samples thus obtained were put to the determination of volatile basic nitrogen and formol titrating nitrogen as mentioned in the previous report.

The determination of pH value was made as follows: 2 g of minced

material was pounded in a mortar with 10 cc of distilled water, allowed to stand for a short time, and the pH value of the supernatant liquid was determined with Toyo pH test-paper (methyl red and phenol red) and its standard table of change of colour which had been corrected with buffer.

Results obtained were as follows:

Table I.

Time (hrs.)	Volatile basic N*		Formol titrating N*		pH	
	Sei-whale meat	Beef	Sei-whale meat	Beef	Sei-whale meat	Beef
0	16.5	21.3	102	52	5.9	6.0
24	128	64.4	211	123	6.5	6.2
48	493	250	616	345	7.4	7.1
72	776	392			7.9	7.1

(* in mg/100 g)

b) Autolysis.

Materials used were the same as in a).

Each material was minced, thoroughly pounded in a mortar, and several samples of 2 g each were placed in glass bottles.

These bottles were corked after the addition of 18 cc each of distilled water saturated with chloroform, and allowed to stand at room temperature. At start and after 48 and 120 hrs., 30 cc each of absolute alcoholic 0.01 *N* H_2SO_4 were added in each bottle to prevent subsequent progress of autolysis. Samples obtained in this manner were put to the determination of the two kinds of nitrogen.

Formol titrating nitrogen.— The sample was transferred to a beaker, heated in a gently boiling water bath for about 15 min., and the supernatant clear liquid was filtered into an Erlenmeyer's flask. 10 cc of distilled water was added to the meat residue in the beaker, heated in a water bath for 5 min., and filtered as before. After the washing of the precipitate was repeated twice more, 1 cc of 20% sulfosalic acid was added to the combined filtrate and washings in the flask. The mixture was allowed to stand overnight, and filtered into a 50 cc volumetric flask. The residue on the filter was washed, and the combined filtrate and washings were diluted to the marking. 20 cc of the solution thus obtained was put to the formol titration.

Volatile basic nitrogen.— 20 cc of the solution obtained above was subjected to the aeration method as described in the previous report.

Results obtained were as follows:

Table II,

Time (Hrs.)	Volatile basic N*		Formol titrating N*	
	Sei-whale meat	Beef	Sei-whale meat	Beef
0	22.8	23.9	101	52.1
48	25.4	32.0	118	82.2
120	28.1	41.0	124	138

(* in mg/100 g)

In concluding this report, the author wishes to express his deepest gratitude to Dr. Tsutomu Maruyama for his kind guidance throughout the experiment and to Prof. Dr. Shichiro Akiya for his kind advices given during the work. Further, the author wishes to thank Mr. T. Tawara and Mr. R. Fukazaaw who helped him in the experiment, and to Mr. R. Nagaura and Mr. T. Nishihara for their assistance in providing the opportunities for obtaining materials for the present experiment.

(Feb. 10, 1948)

On the simultaneous extraction of vitamin A-D and vitamin B_2 complex from the liver of a fin whale (Nagasu-kujira, *Balaenoptera physals L.*).

Tadashi Tawara

Since the discovery of vitamin A in 1913, by Osborne and Mender,[1] studies of vitamin showed extraordinary development. Later, Mellanby,[2] McCollum,[3] Sherman, Pappenheimer[4] and others distinguished between vitamins A and D.

Then Kawakami,[5] Kawai[6] and others confirmed that vitamins A and D existed in the liver of fishes widely and in rich concentrations. Material for vitamin A and D is now almost wholly supplied from the liver of aquatic animals.

It was also discovered that vitamins A and D existed richly in whale liver. Whale liver is now one of the most essential resouces of vitamin A-D oil, as its volume is very large.

In 1926, vitamin B_2 was distinguished from vitamin B, by Goldberger and others,[7] and so-called "Complexity of B_2" was claimed.

Members of B_2 group is now said to contain many factors including the following: lactoflavin (riboflavin), nicotinic acid, vitamin B_6 (adermin), pantothenic acid and other factors. Then, Kuhn,[8] Karrer[9] and others discovered that liver contained a large amount of vitamin B_2 complex.

B_2 complex is richly contained in the liver of aquatic animal, especially, in those of tunny (Maguro; *Thunnus orientalis*), spearfish (Makajiki; *Makaira mitsukurii*), swordfish (Mekajiki; *Xiphias gladius*), bonito (Katsuo; *Katsuwonus vaganus*), fin whale (Nagasu-kujira, *Balaenoptera musculus L.*), humpback (Zato-kujira; *Megaptera nodosa Bonn*), etc.

There are many method of extracting liver oil, principal methods being as follows:

(A) Heating with steam——

Liver is heated with steam, and then the oil separated.

(B) Resolving by standing——

Liver is left standing, putrefied, resolved, and the oil separated.

(C) Vacum method——

Liver is placed in a vacuum kettle, and the oil separated by heating with low pressure steam or hot water.

(D) Refrigeration method——

Liver is pressed while frozen, and the oil separated.

(E) Electrolytic method——

Liver cells are destroyed by passing an electric current, and then the oil separated.

(F) Extraction with organic solvents——

(i) Dry method——

Liver is heated and almost dried. And the oil extracted with organic solvents such as ether.

(ii) Resolving with caustic soda——

Liver is dissolved in caustic soda, and then the oil extracted with organic solvents, or separated by centrifuge.

At present, the last method is almost always employed because the yield of liver oil is very good, and vitamins A and D are extracted almost completely without decomposition.

The present method of preparation of liver oil is limited to the obtaining of vitamin A-D oil, and sacrificing of other effective components which are contained in the liver is unavoidabl.

The author aimed at this point and tried to extract as completely as possible both the vitamins A-D and B_2 from one liver.

Vitamin A is quite stable to alkalis but is sensitive to acids, while vitamin B_2 (lactoflavin) is quite stable in strong mineral acids but is sensitive to alkalis. Vitamin A is sensitive to oxidation, but lactoflavin is quite stable to agent. On account of these conflicting properties, it is difficult to extract both A-D and B_2 completely without decomposition.

Vitamins A and D are fat-soluble vitamins while B_2 complex is water-soluble. The author, therefore, tried to extract first, B_2 complex with water, and then A-D oil from its residue. Extraction of vitamin B_2 complex on various conditions were tested to observe how the quantity of vitamin A changed by various conditions, and to discover conditions under which B_2 complex is extracted completely and decomposition of vitamih A is the least. Vitamin B_2 complex was extracted by various methods and the quantity of lactoflavin in the extract was measured. Its residue was then resolved with caustic soda, the liver oil extracted with ether, and then the

qunntity of vitamin A was measured.

Considerations were given to the effect of air, acids and heat on vitamin A, and to the effect of destruction of cells, and temperature of extraction on Bitamin B_2.

Fin whale liver was used as material.

Experimental

Fin whale liver was minced in a meat-chopper, mixed homogeneously and 100 g of it were used for each experiment.

I. Quantitative analysis

A) Quantitative analysis of vitamin A.

Raw liver or the liver from which B_2 complex had been extracted was taken, about 100 cc of water added and warmed in a water-bath which was later brought to a boil. After the meat had coagulated, solution of 2 g NaOH dissolved in 20 cc of water was added little by little to the coagulated liver with agitation. In the meantime, about 200 cc of water was also added to it, little by little.

After the whole quantity of NaOH solution and water were added, the heating was continued until the granules of liver melted completely and turned into a liquid. This liquid was then allowed to cooled, transferred into a separating funnel, and vitamin A-D oil was extracted five times with 140 cc each of ether. The upper etherial layer was gathered, and washed with water until the washings did not colour to phenolphthalein. After the etherial solution was dried with anhydrous Glauber's salt, ether was distilled off, and vitamin A-D oil was obtained.

The yield of this oil was weighed, and the quantity of vitamin A in 1 g of the oil was measured with spectrophotometer by spectro-graphic method (solvent, chloroform).

$E^{1\%}_{1\,cm}$ 328 millimicron$=1600$[10] was used.

International unit (I. U.) per gram multiplied by the yield of oil gives the total amount of vitamin A in 100 g of liver.

B) Quantitative analysis of vitamin B_2 (lactoflavin)

10 g of acid clay (Fuller's earth) was added to the liquid by which vitamin B_2 was extracted from 100 g of liver. By shaking this mixture vigorously, lactoflavin was adsorbed completely, and then filtered. The adsorbed earth on the filter paper was washed with a small amount of

water, and dried at 60°C. 50 cc of alcohol (94%) was added to the dried earth, the mixture shaken, filterd and the earth again dried at 60°C.

Lactoflavin was eluted from one-tenth quantity of this adsorbed earth with 20 cc of 40% alcoholic 0.25 N-NaOH solution. The residual Fuller's earth was eluted again with 20 cc of 40% alcoholic 0.1 N-NaOH solution, and the two lots of solutions were brought together. This was exactly neutralized with HCl, concentrated at 40°C under a reduced pressure, and alcohol was then distilated off. This was further concentrated to about 10 cc, and was made into a 0.5 N-NaOH solution by the addition of 5 N-NaOH solution.

This solution was measured by Kuhn's method[11] [12] with Pulfrich Stufenphotometer (Filter S. 47; $E^{100\,\gamma/cc}_{1\,cm}=4.75$).

The value thus obtained multiplied by the gives the quantity of lactoflavin (γ) in the extract obtained from 100 g of liver.

II. Extraction of vitamin A-D oil and B_2 complex under various conditions.

(1) Quantative analysis of total vitamin A in 100 g of liver.

Total quantity of vitamin A extracted from 100 g of minced liver by the abovementioned method of resolving with caustic soda, was measured.

Vitamin A	720,000 I. U.

(2) Extraction of vitamin B_2 complex by boiling.

100 g of liver as above was taken. 300 cc of water was added to it, and the solution was brought to pH 5.0 with the addition of phosphoric acid. This solution was boiled in a water-bath for 2 hours, filted with filter-paper while hot. By the above-mentioned method, the quantity of vitamin A in the total amount of residue, and the quantity of lactoflavin in the filtrate were measured. In this case, owing to having boiled in acidity, the amount of vitamin A decreased greatly.

Vitamin A	550,000 I. U.
Lactoflavin	1086 γ

(3) Extraction by boiling in vacuum.

Since vitamin A is oxidized easily in acidity and on heating, the boiling was made under a reduced pressure in order to prevent decomposition. However, the degree of the heating was to such an extent that water in the water-bath was boiling although, it goes without saying that the temperature of liver solution did not reach 100°C. Other conditions were the same as above.

The quantity of vitamin A in this case, was greater, but, on account of insufficient heating, the amount of lactoflavin decreased.

Vitamin A	690,000 I. U.
Lactoflavin	1022 γ

(4) Extraction by digesting.

The liver was digested in order to make the the extraction of vitamin B_2 complex better by destruction of liver cells.

100 g of liver as above, was taken, 300 cc of water and 15 cc of 90% phosphoric acid were added, and then 1 g of saccharated pepsin (J. P.) added with stirring, and kept at 40°C for 2 hours. The digested solution thus obtained was filtered, and the same processes as above were carried out on the filtrate and the residue.

In this case, the extraction of B_2 became somewhat better, but owing to higher acidity the amount of vitamin A obtained was decreased.

Vitamin A	576,000 I. U.
Lactoflavin	1150 γ

(5) Extraction by digesting in vacuo.

The digesting process, in this case, was carried out in vacuo. Other conditions were the same as above.

Vitamin A	690,000 I. U.
Lactoflavin	863 γ

The amount of vitamin A became somewhat better, but, on account of insufficient heating under reduced pressure, the yield of B_2 decreased exceedingly.

(6) Extraction by digesting and boiling.

In order to extract lactoflavin more completely, the digested liquid was boiled for 10 minutes, filtered while hot, and the residue and the filtrate were treated in the same manner as above. The condition of the digestion was the same as for (4).

Vitamin A	456,000 I. U.
Lactoflavin	1788 γ

(7) Extraction by digesting und boiling in vacuo.

In the above process, vitamin A was oxidized greatly because of boiling the solution in strong acidity. Hence the same process was carried out in vacuo.

Vitamin A	675,000 I. U.

Lactoflavin	1563 γ

(8) Extraction by digesting, neutralizing and boiling.

In the above process satisfactory result could not be obtained on the yields of both A and B_2. This must have been due to the fact that vitamin A was boiled in acid, and also to the reduced pressure.

The liver, accordingly, was digested by the method as in (4), then the solution exactly neutralized with NaOH solution, boiled for 10 minutes, and filtered.

Vitamin A	645,000 I. U.
Lactoflavin	1789 γ

(9) Extraction by digesting, neutalizing and boiling in vacuo.

The operations as in (8) was carried out in vacuo.

Vitamin A	703,000 I. U.
Lactoflavin	1406 γ

In all the methods of extractions under various conditions mentioned above, the vacuum process for the extraction of vitamin A brought a good result, but it brought a bad result on B_2. The inferior result of the extraction of B_2 was due to the failure of the temperature to reach boiling under reduced pressure.

Hence, it was thought that if the boiling was carried out under oridinary pressure without air, the result would be better. Carbon dioxide gas was used as an inert gas for this purpose.

---

(10) Extraction by digesting, neutralizing and boiling in carbon dioxide gas.

CO_2 gas was used instead of a vacuum, and all the processes of digestion, neutralization and boiling were carried out in CO_2 gas.

Vitamin A	711,000 I. U.
Lactoflavin	2110 γ

The loss of vitamin A, as a result, was almost negligible, and the extraction of B_2 was better.

(11) Extraction by boiling, digesting, neutralizing and boiling in CO_2 gas.

The liver was coagulated by boiling so as to allow pepsin to act easily on proteins, and cooled to 40°C by adding water, pepsin was then added. All the process were carried out in CO_2 gas. Other conditions were the

same as for (10).

Vitamin A	711,000 I. U.
Lactoflavin	2849 γ

Summary

Conditions under which vitamin A is decomposed least and vitamin B_2 extracted completely from a liver, are as follows:

Minced liver is boiled with water in CO_2 gas, cooled to 40°C by adding water, acidified with phosphoric acid, and the liver substance is digested with protease such as pepsin at about 40°C. After digestion is completed, the solution is exactly neutralized with NaOH, boiled again and filtered. All the above process carried out in CO_2 gas.

Vitamin B_2 is obtained from this filtrate, and vitamin A-D oil from the residue.

In conclusion, the author expresses his cordial thanks to Dr. T. Maruyama and Mr. T. Nakai for their kind advice and encouragement during this study.

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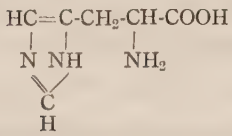
STUDIES ON WHALE BLOOD. I.

On the separation of histidine from whale blood.

Tadashi Tawara

Histidine is an amino acid of the following formula:



This amino acid was discovered in 1896 by Kossel and is found in various proteins, being most abundant in hemoglobin which contains about 11% histidine.

Histidine is one of the most important amino acids for nutrition and it is contained comparatively in a large amount in fish meat. Putrefaction of meat turns histidine into a poisonous histamine. Histidine is used for gastric ulcer.

Two methods of synthesis have been worked out by Pymann.[1,2] The most common material now being employed for hislidine manufacture is blood. There are two principal manufacturing methods, one by precipitating the silver salt in alkaline medium,[3,4,5] and the other using the mercuric salt.[6,7,8]

The author used dried blood of a sperm whale (*Physeter catodon L.*) as material for his experiments.

Cystin, methionine, ornithin, tryptophane and histidine are some of amino acids that precipitate by corrosive sublimate (mercuric chloride). Cystine almost precipitate out in neutral media, while ornitine and tryptophane are not obtained by hydrolysis with hydrochloric acid. Methionine precipitates almost wholly in saturation of sodium chloride around pH 6.0. The author, therefore, undertook the experiments with a special consideration for the removal of methionine.

Experimental

1 kg. Dried blood of sperm whale was hydrolysed with 4 L. 30% hydrochloric acid for 20 hours. After cooling, pigment that precipitated

out (melanine) was removed by filtration and the filtrate was neutralized to pH 2.5 with concentrated sodium hydroxide solution. After standing overnight, the solution was filtered, and the precipitate was washed with a small amount of water. The filtrate and washings were neutralized to pH 6.0 with sodium hydroxide solution and concentrated untill sodium chloride precipitated out. The mixture was cooled and filtered. Methionine can be obtained from this precipitate.

A hot saturated solution of 400 g mercuric chloride was added to the filtrate with stirring and after being left overnight, was filtered. A concentrated solution of sodum carbonate was added slowly, with stirring, until the pH of the solution reached 7.2. After stirring overnight, the supernatant liquid was decanted and the flask filled with water to the original volume. This procedure was repeated several times, and the mixture was finally filtered by a Buchner funnel. From the washings, mercury can be recovered by passing hydrogen sulfide gas.

This precipitate (mercuric salt of histidine) was suspended in 1 L. of water and decomposed by passing hidrogen sulfid gas. This was filtered to remove mercuric sulfide, which was then boiled with water several time to recover histidine adsorbed in the precipitate.

The filtrate and washings from mercuric sulfide were concentrated to about 150 cc, mixed with 300 cc 95% alcohol and stood overnight in an ice chamber. Crude crytstals of histidine monohydrochloride were thus obtained.

The crude product was dissolved in five times its weight of water, and then diluted with twice its volume of 95% alcohol. The yield of recrystallized histidine monohydrochloride was 39 g. This compound melts at 252°C with decomposition.

In conclusion, the author wishes to express his sincere thanks to Dr. T. Maruyama, Mr. H. Okuda and Mr. T. Nakai for their kind guid and advice in this study.

His thnks are also due to prof. Dr. S. Akiya and prof. Dr. T. Ogawa, Tokyo University, for their valuable suggestions.

He is also indebted to Mr. R. Fukazawa for kind assistance during the work.

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Sinus-hairs of the Sei-Whale (Balaenoptera borealis)

by

Junnosuke Nakai and Toshitomo Shida

(Director: Dr. Teizo Ogawa)

Sinus-hairs of a whale possess sensitive tactility like the whiskers of a cat. The root of this hair is surrounded by blood sinus in the connective-tissue layer of the hair follicle. It is a venous sinus with numerous nerves coming into the sinus and twining themselves around the root of the hair.

The whale-bone whales have these characteristic sinus-hairs on their upper and lower beaks, but in dolphins they occur only in their foetus-stage and degenerate after birth.

The lack of sinus-hairs in dolphins is believed, generally, to be due to the food of them, being so large as to be easily visible that tactile hairs become unnecessary, but, the food of the baleen whales is tiny Mysidae and is so difficult to see that they search for the food with these hairs.

In the summer of 1946 and 1947, at Ayukawa, Miyagi Prefecture, we had a chance to study sinus-hairs of Sei-Whales, Balaenoptera borealis, which were caught off the coast of Kinkazan and studies were made both at the whaling station and in the laboratory later. The distribution and number of sinus-hairs macroscopically and some facts on histological preparations have been studied.

D. G. Lillie (1910) merely studied the distribution and number, while A. Japha (1910) devoted himself to the microscopic study of histological preparations.

We observed, at the whaling station, some white, weak hairs of 1 to 2 cm in length occurring on the giant body, generally symmetrically in specific arrangement. The observation at the whaling station was not easy; because of the giant body and of the speedy disposition of it. It was almost impossible to count and examine the hairs of both upper and lower beaks of an individual.

The arrangement and number of these hairs are shown in Table 1 and in Figs. 1 and 2. There are four rows of them on the dorsal surface of the upper beak, from the anterior extremity to the blow-hole, almost 30 hairs at each distance of 20—30 cm. At the anterior end of the lower jaw, there are two rows which occur closely together along the median line at

Individual	upper beak				lower beak			
	right		left		right		left	
	outer row	inner row	outer row	inner row	on the lateral side	at the anterior end	on the lateral side	at the anterior end
No. 1	10	5			9	11		
No. 2			8	4		14		16
No. 3						14		14

Table 1. Number of sinus-hairs of Sei-Whale.

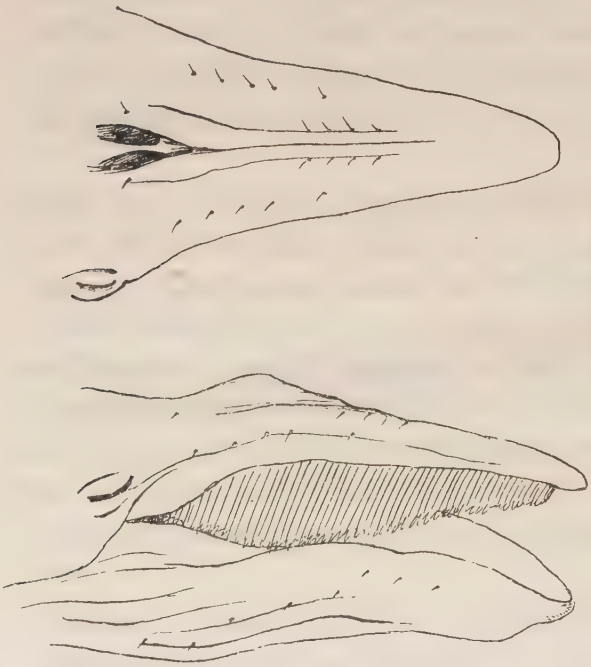

Fig. 1. Arrangement of sinus-hairs on the head of Sei-Whale.

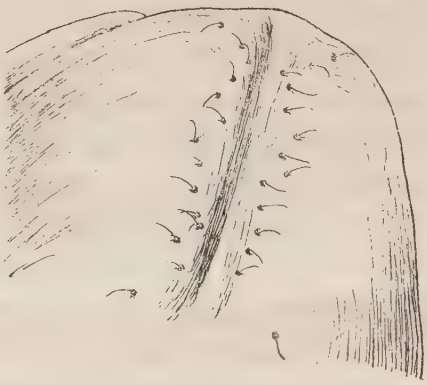

Fig. 2. Sinus-hairs at the anterior end of lower beak.

a distance 1 to 2 cm and contain almost 30 hairs. On each lateral side of lower jaw, there is a row of from 5 to 10 hairs. There are a total of nearly 80 hairs in all which does not greatly differ from reports of the former investigators.

We especially made a study of the hairs at the anterior end of lower beak. We made observations at first by naked eye or with a magnifying-glass inflicting small cuts in the sinus and its neighbouring parts. By this means, we were able to unearth some new facts which seemed to have escaped notice of earlier workers.

When a large blood sinus, surrounding the hair root, was carefully

observed, the skin above it was indented in a funnel shape and became very thin at the point where the hair came out of the sinus, and the sinus extended itself to just beneath the epidermis.

At the bottom of the funnel shaped dent, the thickness of epidermis is only 0.3 mm, but it becomes thicker outward, being 1.3 mm thick at the periphery of the funnel. The corium layer between the epidermis and the blood sinus is only 0.1 mm in thickness.

The sinus-hair is supported in the sinus by many threads from surrounding walls so that the hairwill move freely in all directions when pressure is applied from outside. It is significant that this part of the epidermis where the hair comes out is very thin in order to make this motion easily.

Observations of the cross section of the hair follicle show that this hair is situated not equally distant from inner walls of the sinus, but is eccentric. Furthermore, the inner walls of the sinus are different in shape, such that the side to which the hair shaft is inclined is almost vertical, but the wall on the opposite side forms a curve, of which the central part swells out remarkably (Fig. 3).

The longitudinal diameter of the sinus is almost 12 mm, maximum transverse diameter almost 2 mm. The wall of the sinus is made of dense connective tissue fibers, looking somewhat like the tendon, and is about 1 mm in thickness at the central swollen part. These fibers give off branches radially into surrounding parts of the corium so that the swollen part seems to be strongly pulled outwards.

In the skin of the whale, there is only a thin compact layer of connective tissue between the epidermis and the adipose tissue of the subcutaneous layer. Coarse fiber bundles run in complicated directions through the adipose tissues, but, as no remarkable connective tissue fibers are seen in the neighbourhood of the hair follicle except the above-mentioned radial fibers, the hair follicle lies nearly surrounded by soft adipose tissues.

We thought that there might be a rule between the eccentric location of the hair root in the sinus, shape of the sinus walls, and the inclination of the hair shaft. By a closer observation, it was found that the hair shaft was always inclined towards the side to which the hair root was located nearer. Further observations revealed that the epidermis forming the funnel at the exit of the hair has a gentler slope on the side the hair shaft is inclined

and the other side constitutes an almost perpendicular wall (Fig. 3).

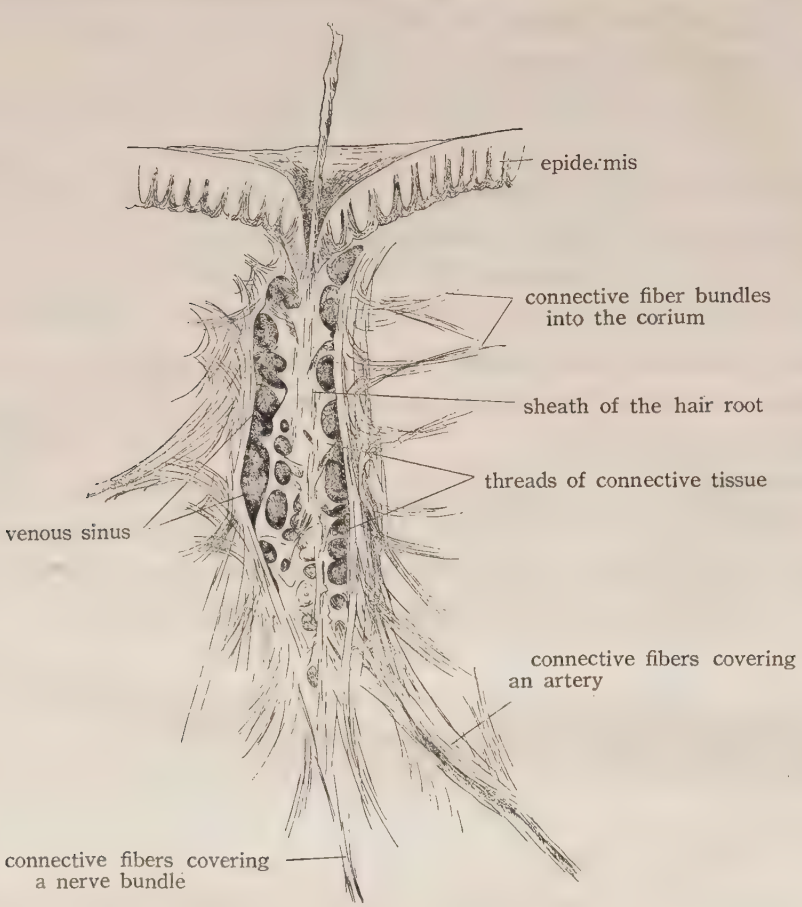
epidermis
connective fiber bundles into the corium
sheath of the hair root
threads of connective tissue
venous sinus
connective fibers covering an artery
connective fibers covering a nerve bundle

Fig. 3 Root of a sinus-hair and its surroundings

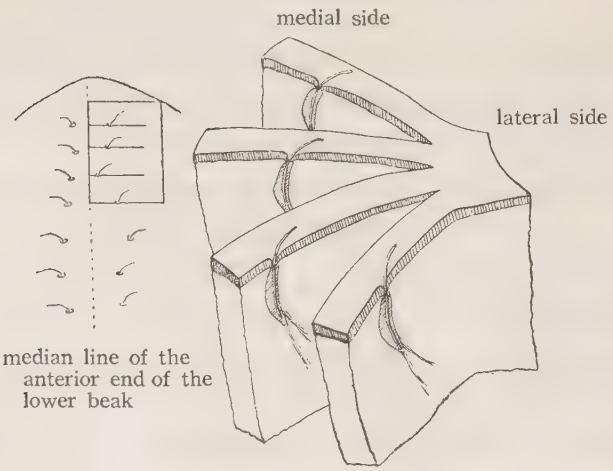

Fig. 4. To show the inclination of sinus-hairs

Therefore, the direction of inclination of the hair shaft seemed to have some intimate correlation to the structure of the sinus. But since we could not make any further generalization on a small piece cut out from the lower jaw, we proceeded then to the examination of the entire tipe of the lower jaw, where each direction in relation to the whole body is easily determinable. It was made clear that the hair was always inclined to the lateral direction (Fig. 4). Therefore, the swollen part of the sinus wall is on the medial side, namely, on the side directed to the median line.

The shaft of the sinus-hair shows no pigmentation and is 0.1—0.15 mm in diameter, being the thinnest at the exit. Its surface is relatively smooth only at the part, where it just comes out of epidermis and its tip has many protrudings and looks like a slender tree trunk with many knots (Fig. 5). While treating the hair under a microscope, it was found that dust adheres easily to the hair shaft. The hair has no marrow; bulbus and papilla of the hair are divided in complex form.

Next, the nerves come into consideration. We observed both in macro

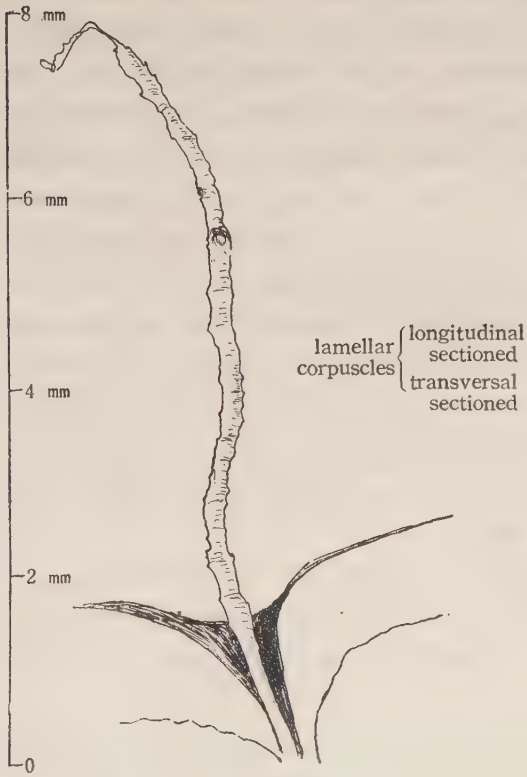

Fig. 5. shaft of a sinus-hair

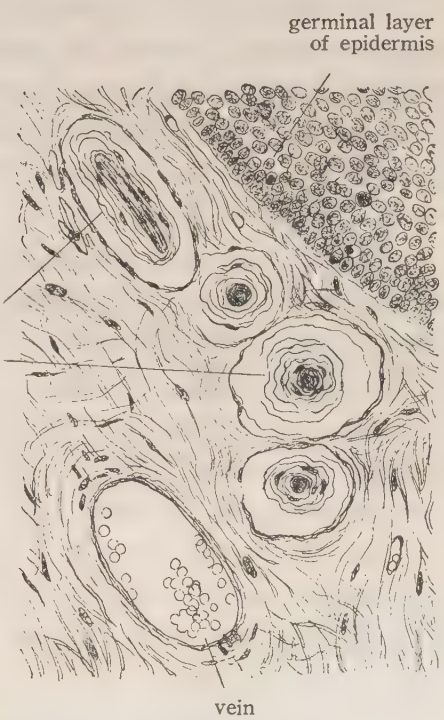

Fig. 6. Lamellar corpuscles in the superficial part of a hair-follicle

and microscopical sections, a relatively thick bundle of nerve fibers ascending almost vertically and it gave off one branch entering the hair follicle from just beneath it and the other branch ascending outside of the follicle.

According to Japha, as several hundreds of myelinated nerve fibers are counted in one sinus-hair, there must be almost 10.000 fibers at the anterior end of the lower jaw, and he quoted in relation to this an interesting story, told by Malm (1866) that when one touched the lips of a Blue whale washed ashore alive near Göteborg in Sweden this giant whale started violently.

We ascertained by microscopic observations that the ascending nerve bundles end in special terminal apparatus, in the outside of the sinus. This apparatus resembles the Pacinian corpuscle, but since the number of lamellae is so few that, it comes nearest to the Herbsti's corpuscle.

They are nearly oval, 60—300 μ in length, 20—150 μ in transverse diameter, and are made of concentric lamellae of connective tissues. The axis of the corpuscle is occupied by a well developed eosinophile core, containing the termination of a nerve fiber (Fig. 6).

The corpuscles are grouped in the following wise: one group about 1 mm immediately beneath the epidermis, on the lateral side of the hair; another group about 1.5 mm in length on the opposite side (median); the third group on the lateral side (same side with the first group) somewhat beneath the second, and also at the height of the hair papilla, where more corpuscles are obviously seen on the median side than on the lateral side.

Further researches are needed as to the distribution of the terminal apparatus and differences in its size according to location. We can, however, assume that this distribution of the corpuscles has a close connection with the functional direction of the hair, when the hair encounters pressure and resistance. Japha also observed that there were especially numerous small terminal corpuscles (about 20×60 μ) in the papillae of corium immediately beneath the epidermis around the hair.

We are endeavouring to throw more light by the method of axis cylinder staining on the distribution of nerve fibers to the corpuscles and to study the characteristic feature of nerve fibers at the point, where the sinus continues into a vein.

The inner surface of the venous sinus, as well as nerves, blood vessels and threads of connective tissues which are all present inside the sinus are

covered by endothelial cells.

According to macroscopic studies, a relatively thick artery, being the size of a thin needle, runs up from the under and lateral direction and enters the sinus through the lower lateral part of the hair follicle, about at the height of a hair papilla. The artery entering the sinus is divided into two, runs upward and becomes more brouched and thinner.

A large amount of blood sent into the sinus from the lower part, runs up and seems to flow out into a vein near the epidermis. The exit is situated on the opposite side to the artery, namely on the median side. The terminal corpuscles are crowded in this side near the exit.

It is especially worthy of notice, in the above-mentioned results, that a rule of direction exists between the sinus-hair and things accompanying it, as a whole. For this reason, the sinus-hair seems to have a greater significance in the life of a baleen whale than it has been assumed as a tool for finding food. We suppose that by use of sinus-hairs the whales might feel the currents of water upon their heads.

Kükenthal was of the opinion that the scanty remaining hairs of whales are merely a reduced organ, as they have no sebaceous glands. But it is the common property of the skin of whales that it lacks not only the sebaceous glands, but also the sweat glands. We are of the opinion, the sinus-hairs of whales seem to be a very important sensory organ for the life of them.

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